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Relationship Between Human Papillomavirus Infection and Overexpression of p53 Protein in Cervical Carcinomas and Lymph Node Metastases

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Overexpression of p53 protein is common in cervical carcinoma. We investigated archival biopsies from 26 cervical cancer patients (24 with available lymph nodes) to determine the relationship between p53 overexpression and HPV infection at the cervix and lymph nodes. Twelve cervical carcinoma patients had p53 protein in cervical biopsies detectable by immunohistochemistry using monoclonal antibody DO-1, and 22 were positive for HPV DNA in polymerase chain reaction assays (16 contained HPV-16; 3, HPV-18; and, 3 HPV-X). Seven cervical cancer patients had one or more lymph nodes positive for p53 protein, and all but one of these were concordantly p53 positive at the cervix. However, detection of p53 protein in cervical biopsies was predictive neither of the expression of p53 at draining lymph nodes ($P > 0.1$) nor of the occurrence of metastases ($P > 0.1$). Fourteen patients were positive at one or more lymph nodes for HPV DNA. Cervical positivity for HPV DNA was associated significantly with concordant HPV positivity at the lymph nodes ($P = 0.039$) and was predictive of metastases ($P = 0.019$). There was no association between positivity for p53 and for HPV DNA at primary cervical carcinomas or at the lymph nodes (all $P > 0.1$). We conclude that, although detectable p53 protein is a common feature of cervical carcinomas, it is not predictive of metastases and is independent of HPV infection. ***J. Med. Virol. 53:111–117, 1997.***

KEY WORDS: p53 overexpression; human papillomavirus; cervical carcinoma; metastases

INTRODUCTION

Infection with human papillomavirus (HPV) types HPV-16 and -18 (and also, but less frequently, types HPV-31, -33, -35 -45, -51, -52, and -56) is considered to confer a high risk for cervical carcinoma [deVilliers, 1992; zur Hausen, 1994]. Indeed, cervical infection with these high-risk HPVs may be prognostic for the occurrence of lymph node metasteses in cervical cancer [Fuchs et al., 1989]. The oncogenic activity of high-risk HPVs is largely dependent on the binding of HPV E6 protein to p53, which results in the ubiquinone-dependent degradation of p53 [Scheffner et al. 1990].

p53 has two biological activities that protect against cancer: down-regulation of cell cycle progression to G_1 and initiation of apoptosis. Consequently, loss of p53

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TABLE I. Summary of Results[a]

	Cervix		Lymph node[b]		
Patient	DO-1	HPV	Histology	DO-1[b]	HPV[b]
1. CaCx					
9	+++	16	2/2	$2/2^{++/++}$	$1/2^{(16)}$
27	++++	16	3/3	$1/2^{++}$	$3/3^{(16)}$
12	++	16	1/1	$1/1^{+++}$	$1/1^{(16)}$
15	+	16	4/4	$1/2^{++}$	$1/4^{(16)}$
25	++	16	2/2	0/2	$2/2^{(16)}$
22	+++	X	1/1	$1/1^{++}$	0/1
2	++++	X	1/1	0/1	0/1
30	++++	16	0/1	0/1	0/1
29	+	16	0/1	0/1	0/1
17	++++	18	0/1	0/1	$1/1^{(18)}$
6	+++	18	0/2	$2/2^{+++/+++}$	$1/2^{(18)}$
16	++	—	0/1	0/1	0/1
3	−	16	2/2	0/2	$2/2^{(16)}$
4	−	16	1/1	0/1	$1/1^{(16)}$
10	−	16	4/4	0/4	$4/4^{(16)}$
13	−	16	2/2	0/2	$1/2^{(16)}$
18	−	16	1/1	0/1	$1/1^{(16)}$
26	−	16	1/1	$1/1^{++}$	$1/1^{(16)}$
11	−	16	1/1	0/1	0/1
24	−	16	NA	NA	NA
19	−	16	NA	NA	NA
23	−	18	0/1	0/1	0/1
28	−	X	0/1	0/1	0/1
5	−	—	0/1	0/1	0/1
8	−	—	0/1	0/1	0/1
20	−	—	0/1	0/1	0/1
2. CIN					
31	+++	16	NA	NA	NA
33	++	16	NA	NA	NA
32	−	16	NA	NA	NA

[a]CaCx, cervical carcinoma; CIN, cervical intraepithelial neoplasia; HPV, results of PCRs (16, positive for HPV-16 DNA; 18, positive for HPV-18 DNA; X, positive for HPV DNA using consensus primers but negative for types HPV-16, -18, -31, and -33); DO-1, result of immunohistochemistry using mAb DO-1 (number of crosses represents the relative numbers of cells stained or, —, no staining detected).
[b]Lymph node histology: number of metastatic lymph nodes over the total number examined. HPV, HPV DNA in lymph nodes as number positive over number tested (HPV type indicated in superscript parentheses); DO-1, number of lymph nodes stained by mAb DO-1 over the number tested, positive stains graded by the number of crosses; NA, not available.

from allelic deletion or somatic mutation of the Tp53 gene, or HPV-induced degradation, may be pivotal in the development of several malignancies [Lane, 1994; Scheffner et al., 1990]. Indeed, some cervical carcinoma cell lines can be differentiated on this basis into those that harbour Tp53 mutations and others, which are infected with high-risk HPVs and contain native Tp53 [Crook et al., 1991, 1992]. Generally, however, mutation of Tp53 is infrequent, whereas overexpression of p53 protein is common in cervical carcinomas [Kessis et al., 1993a,b; Park et al., 1994; Bushby-Earle et al., 1994; Bartek et al., 1991; Helland et al., 1993]. Because overexpression of p53 and infection with high-risk HPVs are both independently associated with cervical carcinoma, we investigated the relationship between these factors, primarily to determine whether the coexistence of these factors is prognostic for lymph node metasteses in cervical cancer.

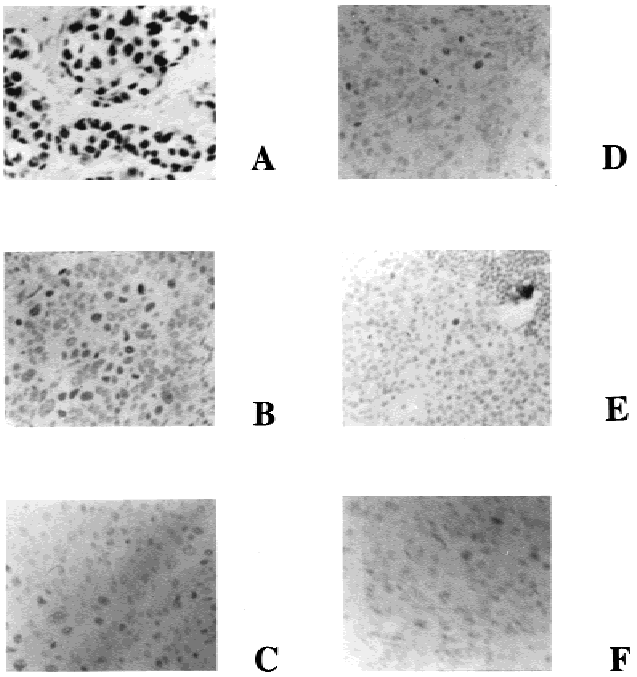

Fig. 1. Examples of immunohistochemical staining of p53 protein with mAb DO-1. Breast carcinoma (+++++; **A**); sections scored ++++ (**B**), +++ (**C**), ++ (**D**); + (**E**), and negative (**F**).

MATERIALS AND METHODS

Clinical Samples

Formalin-fixed, paraffin-embedded, cervical biopsies from 26 patients with squamous cell cervical carcinomas attending St. Thomas' Hospital, London, were investigated (Table I). Thirty-seven lymph node biopsies were available from 24 patients (27 metastatic nodes from 14 patients and, 10 uninvolved nodes from 10 patients). Cervical biopsies from 3 patients, 2 with cervical intraepithelial neoplasia (CIN)-II and 1 with CIN-III (case 33), were also studied. Patients had a mean age of 44.6 (range 17–65) years.

Indirect Immunohistochemistry

Five micrometer sections (each cut with fresh disposable microtome knives) were fixed to poly-L-lysine (Sigma Ltd., Poole, U.K.)-coated slides, dewaxed, and rehydrated. Endogenous peroxidase activity was blocked with 3% (v/v) hydrogen peroxide for 10 min at room temperature (RT). Sections were microwaved, incubated with 5% (v/v) normal rabbit serum in Tris-buffered saline (TBS) for 20 min at RT, then washed with TBS. Sections were incubated with a murine monoclonal antibody (mAb) DO-1 (at an optimal concentration of 1/200 in TBS; Oncogene Ltd.) for 60 min at RT, then again washed with TBS.

Sections were immersed next in biotinylated rabbit antimouse immunoglobulin (Dako Ltd., Glostrup, Denmark; at 1/300 in TBS for 30 min at RT), washed, and then placed in Strept-ABComplex (Dako Ltd.) for 30 min at RT. Sections were washed in TBS, and bound

TABLE II. HPV DNA in, and DO-1 Staining of, Cervical Biopsies[a]

	DO-1+	HPV+	HPV-16+	HPV-18+	HPV-X+
CaCx	46 (12/26)	85 (22/26)	62 (16/26)	11.5 (3/26)	11.5 (3/26)
2B	40 (2/5)	80 (4/5)	80 (4/5)	0 (0/5)	0 (0/5)
2A	66.6 (2/3)	100 (3/3)	33.3 (1/3)	33.3 (1/3)	33.3 (1/3)
1B	54.5 (6/11)	91 (10/11)	63.6 (7/11)	9.1 (1/11)	18.2 (2/11)
1A	20 (1/5)	80 (4/5)	60 (3/5)	20 (1/5)	0 (0/5)
CIN	66.6 (2/3)	100 (3/3)	100 (3/3)	0 (0/4)	0 (0/3)

[a]CaCx, patients with cervical carcinoma; CIN, patients with cervical intraepithelial neoplasia; DO-1+, immunohistochemical detection of p53; HPV+, positive for any HPV DNA; HPV-16+, positive by PCR and Southern blot for HPV-16 DNA; HPV-18+, positive by PCR for HPV-18 DNA; HPV-X+, MY09/MY11 positive but negative for HPV-16, -18, -31 or -33 DNA. Results are expressed as percentages of those cases tested who were positive in any particular assay; in parentheses are the actual numbers of patients positive over the number tested. Two of the 26 cancer patients were not clinically staged.

antibody was visualised with diaminobenzidine substrate (Sigma Ltd.) for 10 min at RT. Finally, sections were rinsed in water, lightly counterstained with Meyers' haemotoxylin (BDH Ltd.), dehydrated, and mounted in DPX resin (BDH Ltd.). Formalin-fixed, paraffin-embedded sections from a breast carcinoma (which expresses p53) and from normal skin were used as positive and negative controls, respectively. Sections were scored by visual inspection according to the percentage of carcinoma cells stained by mAb DO-1 in the most densely stained area observed. This ranged from +++++ for the breast carcinoma, representing 100% of carcinoma cells stained; to ++++, 50–30%; to +++, 30–2%; to ++, 2–0.1%; to +, greater than 0.01%; to, −, less than one positive cell/10,000 carcinoma cells/no positive cells detected (see, e.g., Fig. 1).

Polymerase Chain Reactions

One 10 μm section per patient was dewaxed with octane, followed by ethanol washes. Cellular material was pelleted by centrifugation and digested with proteinase K [Wright and Manos, 1990] and investigated by polymerase chain reactions (PCRs).

HPV consensus PCR: MY09/11 HPV consensus primers were used [Manos et al., 1989; Pakarian et al., 1994; Cavuslu et al., 1996]: PCR cycle times were doubled in this PCR, and all others, as recommended for amplification of HPV DNA from fixed samples [Wright and Manos, 1990].

HPV-16 PCR and Southern blots: HPV-16 E7 PCRs were used [Kaye et al., 1994; Cavuslu et al., 1996], and Southern blots of PCR products using a digoxigenen end-labelled HPV-16 E7 DNA probe and an alkaline phosphatase-based detection system (Boehringer-Mannheim Ltd.) were performed.

HPV-18, -31, and -33 PCRs: HPV-18, -31, and -33 PCRs were conducted as described previously [van den Brule et al., 1990; Cavuslu et al., 1996].

β-Globin PCRs: To confirm that sufficient quantities of DNA were present, and that nonspecific PCR inhibitors were absent, samples were analysed for β-globin DNA in PCRs using PCO-3 and PCO-4 primers [Saiki et al., 1986].

All PCRs used "hot starts" with paraffin wax (BDH Ltd.), and rigorous precautions to prevent contamination were undertaken [Muir et al., 1993]. Three human cell lines were used as controls in PCR assays (using 1,000 cells per reaction): SiHa cells, which contain HPV-16 [El Awady et al., 1987]; HeLa cells, which contain HPV-18 DNA [Schneider et al., 1986]; and A431 cells, which have no HPV DNA. Positive controls for HPV-31 and -33 PCRs were amplicons derived from clinical samples [Pakarian et al., 1994]. Each PCR experiment included one negative (distilled water or A431 cells) and one positive control (see above) per eight samples.

Statistical Analyses

χ^2 Tests and Fisher's exact test (Fet) were used.

RESULTS

Cervical Biopsies

p53 Expression. Twelve of twenty-six (46.1%) cancer patients were DO-1 positive, but this was unrelated to the staging of carcinomas (Tables I, II). Four sections were considered ++++, 3 +++, 3 ++, and 2 +. DO-1 staining of the stratum basale was detected in 2 of 3 (66.6%) patients with CIN (cf. DO-1 staining in cervical carcinomas: $\chi^2 = 0.45$, $P > 0.1$). In all assays, nuclei of cancer cells in breast carcinoma sections were densely stained, whereas normal skin was not.

HPV DNA. Twenty-two (85%) carcinoma and three (100%) CIN patients were positive for HPV DNA ($\chi^2 = 0$, $P > 0.1$). HPV prevalences were not associated with cancer stage, nor were there significant differences between the detection rates of any HPV between carcinoma and CIN patients (Table II). HPV-16 was detected in 16 (62%) cancers and 3 (100%) CIN lesions, HPV-18 in 3 (11.5%) carcinomas; HPV-31 and HPV-33 were not detected in any lesions, and HPV-X (i.e., positive by HPV consensus PCR but negative for HPV-16, -18, -31, or -33 DNA) was found in 3 (11.5%) carcinomas. All cervical (and lymph node) samples were β-globin positive by PCR, and all HPV PCRs produced amplicons from positive, but not negative, controls (data not shown).

Correlation between p53 expression and presence of HPV DNA. Combined negativity for DO-1 staining and HPV DNA was uncommon (3 patients; 11.5%); 11 patients (42.3%) were DO-1 negative but

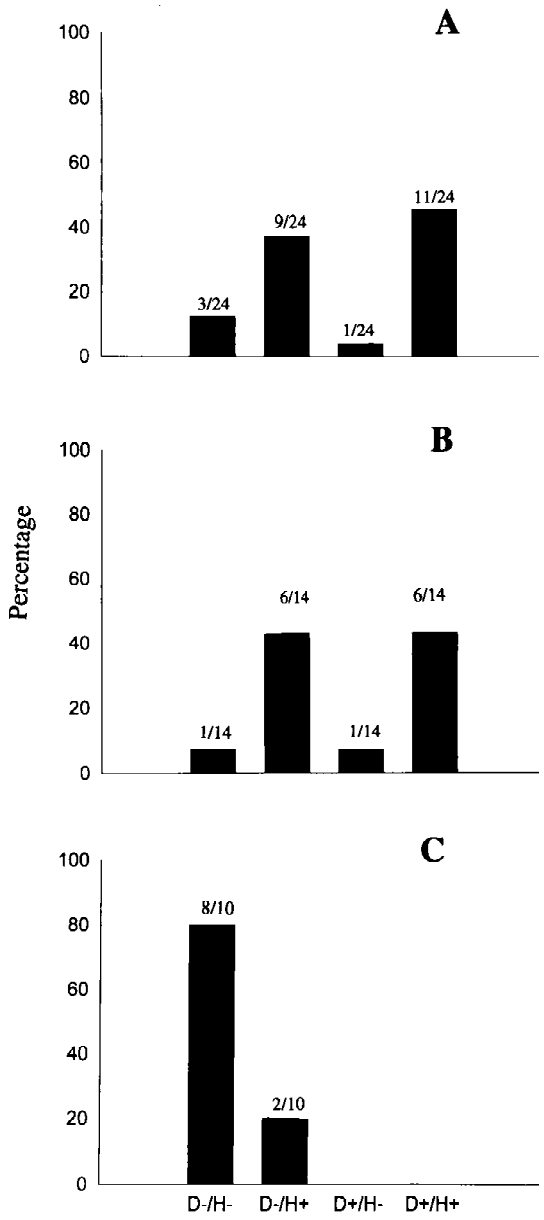

Fig. 2. Relationship between the presence of HPV DNA and immunoreactivity with mAB DO-1. **A:** Primary cervical carcinomas and corresponding metastatic (**B**) or uninvolved (**C**) draining lymph nodes. Results are expressed as percentage of patients in the following categories: D-/H-, negative for HPV DNA and D0-1 staining; D-/H+, negative with mAb DO-1 but positive for HPV DNA; D+/H-, positive with DO-1 but negative for HPV DNA; and D+/H+, positive for DO-1 staining and for HPV DNA. In the case of lymph node results (B,C), if one or more node was positive for the above-mentioned characteristics, the patient was considered positive.

HPV DNA positive; DO-1 positivity in the absence of HPV was rare (1 patient, 3.8%); and 11 (42.3%) patients with carcinomas were concordantly positive for DO-1 staining and for HPV DNA (Table I). There was no significant association between DO-1 positivity and HPV DNA in cervical biopsies from these 26 cancer patients ($\chi^2 = 0.85$, $P > 0.1$) or the subgroup of 24 patients for whom lymph nodes were available ($\chi^2 = 1.2$, $P > 0.1$; Fig. 2A). Among patients with CIN, 1 (33.3%) was positive for HPV DNA but not for DO-1, and 2 (66.6%) were positive for both HPV DNA and DO-1 (Table I).

Lymph Nodes

p53 Expression. Thirty-seven nodes from twenty-four cancer patients were studied: Three nodes contained insufficient tumour and were thus excluded. Nine nodes from seven (29.2%) patients were DO-1 positive. Seven of fourteen (50%) patients with metastases and 0 of 10 (0%) with uninvolved nodes had one or more DO-1-positive node; this difference was significant (Fet $P = 0.019$). Two cancer patients (cases 15 and 27, each with two metastases) had one node positive and one negative for DO-1 staining. Three nodes (from 2 patients) were scored +++, and 6 nodes (from 5 patients) ++.

HPV DNA. Twenty-one lymph nodes from fourteen cancer patients contained HPV DNA; 18 nodes contained HPV-16 and 3 HPV-18 (Table I). HPV was detected in 19 of 27 nodes (70.3%) from 12 of the 14 patients with lymph node metasteses and in 2 of 10 uninvolved nodes (20%) from 2 of 10 patients; this difference was significant (Fet $P = 0.017$).

Concordance of p53 expression and HPV DNA. There were no significant associations between DO-1 and HPV DNA positivity at one or more lymph nodes ($\chi^2 = 3.05$, $P > 0.5$) or for those with metastatic ($\chi^2 = 0$, $P > 0.1$; Fig. 2B) or with uninvolved lymph nodes ($\chi^2 = 0$, $P > 0.1$; Fig. 2C).

Association Between Cervical and Lymph Node Findings

DO-1 positivity at the cervix was not predictive for the occurrence of lymph node metastases (8 of 14 patients vs. 4 of 10 with uninvolved nodes; $\pm^2 = 0.68$, $P > 0.1$), of concordant DO-1 positivity at one or more lymph node ($\chi^2 = 5.04$, $P > 0.1$), or of DO-1 positivity in metastatic ($\chi^2 = 4.7$, $P > 0.1$) or uninvolved ($\chi^2 = 1.11$, $P > 0.1$) lymph nodes. One patient (case 26) was DO-1 positive at a metastatic node but negative at the cervix. There was no significant difference between those with strong (++++ or +++, n = 7) or weak (++ or +, n = 5) cervical DO-1 staining with respect to the subsequent detection of DO-1 positivity in lymph nodes (n = 4 and n = 2, respectively; $\chi^2 = 0.05$, $P > 0.1$).

HPV DNA positivity at the cervix was associated with lymph node metastases (14 of 20 patients with HPV-positive cervices had metastases, whereas 0 of 4 with HPV-negative cervices had metastases; Fet $P = 0.019$) and with HPV positivity at lymph nodes (Fet $P = 0.039$). One patient (case 2) who was positive for HPV-X DNA at the cervix had HPV-18 at the lymph node; in all other cases, the HPV type detected at the lymph node accorded with that at the cervix for individual patients. Six patients with HPV-positive cervices, but negative metastases, were retested with the same result; in addition, histological reassessment of sections confirmed that carcinoma was present in sec-

TABLE III. p53 Overexpression in Some Malignant and Premalignant Lesions[a]

Lesion	Overexpression	Ab	Reference
Renal cell Ca	32/100 (32)	DO-7	Bot et al., 1994
Renal adenocarcinoma	41/124 (33)	CM-1	Lipponen et al., 1994
Oral dysplasia	15/27 (55)	PAb-1802 & 421	Kaur et al., 1994
Oral Ca	24/32 (75)	PAb-1801 & 421	Kaur et al., 1994
BCLL	9/15 (60)	PAb-240 & 1801	Aguilar-Santelises et al., 1994
NHL	10/45 (22)	DO-7	De Re et al., 1994
Prostate Ca	2/19 (10.5)	DO-7	Moyret-Lalle et al., 1995
Endometrial Ca	23/139 (17)	DO-7	Inoue et al., 1994
Normal	5/8 (62.5)	S206-120	Jeffers et al., 1994
CIN-I	5/13 (38)	S206-120	Jeffers et al., 1994
CIN-II	3/14 (21)	S206-120	Jeffers et al., 1994
CIN-III	13/22 (59)	S206-120	Jeffers et al., 1994
Cervical Ca	2/55 (3.6)	CM-1)	Ngan et al., 1994
Cervical Ca	19/22[b] (86)	Pab-240	Cooper et al., 1993
CIN	3/81 (4)	CM-1	Pollanen et al., 1993
Cervical Ca	93/192 (48.4)	CM-1	Oka et al., 1993
Cervical Ca	34/50[b] (68)	CM-1	Helland et al., 1993
Cervical Ca	12/26 (46.1)	DO-1	This study
Cervical Ca LN1	7/14 (50)	DO-1	This study
Cervical Ca LN2	0/10 (10)	DO-1	This study

[a]BCLL, B-cell chronic lymphocytic leukemia; NHL, HIV-1-associated non-Hodgkin's lymphoma; Ca, carcinoma; LN1, patients with metastatic or, LN2, uninvolved lymph nodes draining from a primary cervical carcinoma; CIN, cervical intraepithelial neoplasia; Ab, antibody used for detection of p53 overexpression. The results of these studies are summarised as the number of patients positive over the total number tested and, in parentheses, the percentage positivity. MAbs DO-1 and DO-7 recognise amino acids (aa) 1–45 of p53 and have essentially identical staining properties to the polyclonal antiserum CM-1. In contrast pAb-1801 recognises aa 45–91, pAb-240 aa 161–220, and pAb-421 aa 370–378 [Vojtesek et al., 1992].
[b]HPV-16-positive patients.

tions immediately before and immediately after the one analysed for HPV DNA.

DISCUSSION

In this study we found that both p53 expression and HPV DNA could be detected in many cervical carcinomas and draining lymph nodes and that the patterns of expression of these two factors were similar in primary cancers and in metasteses (Fig. 2). However, these factors were independent statistically of one another at both sites (both $P > 0.1$). Only HPV DNA positivity at the cervix was significantly associated with lymph node metastases ($P = 0.039$). A lack of correlation between p53 expression and HPV infection has also been reported in cervical dysplasia and cervical and bladder carcinomas but has not previously been studied in lymph node metastases [Mittal et al., 1995; Jeffers et al., 1994; Kamel et al., 1995].

p53 Expression was found in 46% of cervical cancers and, at one or more lymph nodes, in 29% of patients. p53 Was common among patients with metastases (50% had one or more positive node) and was not detected in uninvolved lymph nodes ($P = 0.019$). This prevalence of p53 expression in cervical carcinoma is similar to that reported by Oka et al. [1993], but contrasts with other studies in which wide ranges have been documented (Table III). p53 Expression at the cervix was also detected in CIN lesions, as has been noted by others (Table III), and overall the number of cancer cells stained by DO-1 in positive cases was low (less than 5% of malignant cells).

mAb DO-1 recognises native and mutant p53 [Vojtesek et al., 1992]. To investigate whether mutant p53 proteins were expressed, sections were stained with mAb PAb-240, which recognises mutant p53 [Vojtesek et al., 1992]. Whereas the positive control was intensely stained, none of the test sections was positive (data not shown). This could be explained by the fact that PAb-240 binds only weakly to mutant p53 in archival material [Vojtesek et al., 1992]. Alternatively, it is possible that overexpression of p53 represents the accumulation of native p53 protein or even degradation products that bear the antigen recognised by mAb DO-1.

HPV DNA was detected in 85% of cervical cancers and (at one lymph node or more) in 58.3% of patients. Among the cervical carcinomas, HPV-16 was the most prevalent genotype (62%); HPV-18 was found in 11.5% and HPV-X in 11.5%. Whereas HPV-X could reflect the presence of low-risk HPVs (e.g., types 6 or 11), it is most probable that these cases represent other high-risk HPVs. In one instance (case 2), HPV-X was detected at the cervix but HPV-18 at the metastatic lymph node; this may reflect the greater sensitivity of the consensus HPV PCR compared to the PCR for HPV-18.

Insofar as the PCRs used were sensitive [Cavuslu et al., 1996], detection of HPV-negative metastases from patients with HPV-positive tumours was surprising, though again this has been noted by others [Fuchs et al., 1989; Yanuck et al., 1991; Crook et al., 1992; Mvula et al., 1994]. The explanations for such cases are unknown but might include genetic heterogeneity or biclonal origin of metastatic tumour cells [Beyer-Finkler et al., 1995]. We also found HPV DNA in uninvolved

nodes from 2 patients with HPV-positive cervical carcinomas. This might be explained by micrometastases missed by histological assessment or contamination of lymph nodes with tumour cells during surgical removal [Nawa et al., 1993; Ikenberg et al., 1994].

It is concluded that detectable HPV DNA and p53 protein expression are common in cervical carcinomas, that no significant relationship exists between HPV DNA and detectable p53 protein in cervical cancers or metastases, and that only HPV infection at the cervix is associated with the occurrence of metastases.

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